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## REVIEW

## Neutrophil Extracellular Traps in Inflammatory Bowel Disease: Pathogenic Mechanisms and Clinical Translation



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## SUMMARY

Neutrophils play a key role in gut inflammation. Our review focuses on a distinct effector mechanism, the ability of neutrophils to form extracellular traps as a potential pathogenic factor and a therapeutic target for translation in inflammatory bowel disease.

The Inflammatory Bowel Diseases (IBD), Ulcerative Colitis (UC) and Crohn's Disease (CD) are characterised by chronic non-resolving gut mucosal inflammation involving innate and adaptive immune responses. Neutrophils, usually regarded as first responders in inflammation, are a key presence in the gut mucosal inflammatory milieu in IBD. Here, we review the role of neutrophil extracellular trap (NET) formation as a potential effector disease mechanism. NETs are extracellular webs of chromatin, microbicidal proteins and oxidative enzymes that are released by neutrophils to contain pathogens. NETs contribute to the pathogenesis of several immune-mediated diseases such as systemic lupus erythematosus and rheumatoid arthritis; and recently, as a major tissue damaging process involved in the host response to severe acute respiratory syndrome coronavirus 2 infection. NETs are pertinent as a defence mechanism at the gut mucosal interphase exposed to high levels of bacteria, viruses and fungi. On the other hand, NETs can also potentiate and perpetuate gut inflammation. In this review, we discuss the broad protective vs. pathogenic roles of NETs, explanatory factors that could lead to an increase in NET formation in IBD and how NETs may contribute to gut inflammation and IBD-related complications. Finally, we summarise therapeutic opportunities to target NETs in IBD. (*Cell Mol Gastroenterol Hepatol* 2021;12:321–333; <https://doi.org/10.1016/j.jcmgh.2021.03.002>)

**Key Words** IBD, UC, CD, Neutrophils, Inflammation, Immunology, Neutrophil Extracellular Traps.

Ulcerative colitis (UC) and Crohn's disease (CD) are 2 clinical entities that define the inflammatory bowel diseases (IBD). Both are chronic immune-mediated conditions with rapidly increasing global incidence.<sup>1</sup> Although displaying different clinical features, they share a non-resolving pattern of gut mucosal inflammation. Current therapeutic approaches are focused on inhibiting the aberrant gut inflammation and immune response. For example, blocking inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin (IL)-23, the migration of leukocytes with anti- $\alpha 4\beta 7$  integrin therapies, T cell

responses with thiopurines, and downregulating general inflammation with glucocorticoids. Notwithstanding recent therapeutic advances in IBD, primary treatment failure is common, and complete mucosal healing is achieved in 50% of patients with moderate-to-severe IBD. Hence, a creative appraisal encompassing distinct mechanistic pathways is necessary in the design of future treatment strategies beyond mitigating downstream inflammatory responses.<sup>2</sup>

Although prominently featured in the inflamed IBD mucosa, the translational potential of targeting the damaging effects of neutrophils is relatively understudied. A notable inflammatory process driven by neutrophils is mediated via their ability to form neutrophil extracellular traps (NETs). NETs comprise of a scaffold of DNA laced with histones and cytotoxic neutrophil-derived proteins and are released by neutrophils during infection and inflammation to contain invading microbes as a protective response.<sup>3–5</sup> NET components are indiscriminately cytotoxic and proinflammatory and thus can play an active role in a wide range of pathologies involved in autoimmunity, thrombosis, and cancer (Figure 1).<sup>6</sup> Here, we focus on the role of the neutrophils and how their ability to form NETs presents an area for clinical translation in IBD.

## The Role of Neutrophils in IBD

Neutrophils are the most abundant immune cell, constituting approximately 70% of leukocytes in human blood and are regarded as first responders of the innate immune system. Neutrophils are best known for their rapid recruitment to sites of infection or tissue damage to contain pathogens.<sup>7</sup> Neutrophils undergo apoptosis and efferocytosis upon completion of their tasks to allow the

**Abbreviations used in this paper:** AAV, antineutrophil cytoplasmic antibody-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; CD, Crohn's disease; DDIT4, DNA damage inducible transcript 4; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloprotease; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; mtNET, mitochondrial neutrophil extracellular trap; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PMA, phorbol-12-myristate-13-acetate; PR3, proteinase 3; RA, rheumatoid arthritis; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TLR, Toll-like receptor; TNF, tumor necrosis factor; UC, ulcerative colitis.



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resolution of inflammation, repair, and a return to normal tissue function.<sup>8-10</sup> Persistent activation and overexuberant recruitment of neutrophils are common features in many inflammatory diseases. Neutrophils produce a wide variety of chemokines, cytokines, and leukotrienes, allowing them to recruit and activate a broad spectrum of immune cells.<sup>11,12</sup> Neutrophils also directly cause tissue damage by releasing proteases such as matrix metalloproteases and neutrophil elastase (NE),<sup>13,14</sup> as well as from oxidative burst which directly disrupts cell membranes.<sup>15</sup>

Neutrophils play an important role in IBD gut inflammation. They produce high levels of reactive oxygen species (ROS) that cause epithelial barrier damage and can activate redox-sensitive inflammatory pathways.<sup>16</sup> They also released a host of proteases, proinflammatory cytokines and mediators such as IL-8, TNF- $\alpha$ , and leukotriene B<sub>4</sub> that damage the epithelial barrier and recruit monocytes and more neutrophils to the gut.<sup>17,18</sup> Neutrophil infiltration correlates with disease activity in IBD<sup>19-23</sup> and is a widely used and reliable component of UC disease scoring systems.<sup>24</sup> Calprotectin ( $\leq 100$  A8/9), the most widely used biomarker in IBD, constitutes up to 60% of neutrophil cytosolic proteins.<sup>25,26</sup> In addition, many more neutrophil-derived IBD biomarkers have been proposed,<sup>27-29</sup> and there are several examples of neutrophil-targeted therapies. Although a neutrophil response is strongly featured in UC,<sup>30</sup> in CD, neutrophil function may be impaired with reduced migration, superoxide production, and phagocytic functions.<sup>31,32</sup> In contrast to UC, reduced neutrophil accumulation and delayed bacterial clearance is observed at sites of injury within the gut and systemically in CD.<sup>33</sup> This suggests that the development of chronic inflammation and granuloma formation is a consequence of an impaired innate immune response in CD.<sup>33,34</sup> In this regard, similar features are seen in chronic granulomatous disease and glycogen storage disease that are characterized by deficient neutrophil responses.<sup>32,35</sup>

## NET Formation

Brinkmann et al<sup>3</sup> first discovered NETs upon stimulating neutrophils with IL-8, phorbol-12-myristate-13-acetate (PMA), or lipopolysaccharide (LPS). NETs prevent the spread of a range of pathogens<sup>5</sup> by trapping them in an environment of microbicidal components and prevent fungal growth as has been demonstrated with *Candida albicans*.<sup>4</sup> NETs contain granule proteins, histones, cytoplasmic proteins, and notably, calprotectin, which is important in antifungal defence.<sup>4</sup> Extracellular traps are an effective and highly conserved defence mechanism.<sup>36</sup> They have been reported in teleosts,<sup>37</sup> chickens,<sup>38</sup> crabs, and mussels,<sup>39</sup> and in plants as part of the root tip resistance to fungal infection,<sup>40</sup> and many pathogens have evolved mechanisms to repulse<sup>41</sup> or degrade them.<sup>42,43</sup> Given the proximity of the gut mucosa to the microbiota, the role of NETs is of particular importance in IBD as an integral component of the host defense (Figure 2).

Although interest in NETs grows, a refined consensus on NET mechanisms and classification is lacking.<sup>44,45</sup> From a recent expert review,<sup>44</sup> a few aspects are noteworthy; first

NETosis does not cover all forms of NET release and the term NET formation is preferred. Second, there are many in vivo NET stimulants including bacteria, fungal hyphae, cytokines, immune complexes, and activated platelets. Third, there are 2 broad mechanistic pathways for NET formation: late lytic, occurring 2–4 hours poststimulus, which is most commonly induced by PMA and results in neutrophil death, and early nonlytic, which occurs 5–60 minutes poststimulus induced by, for example, Toll-like receptors (TLRs) responding to pathogen associated molecular patterns and not resulting in neutrophil death. However, the mechanisms underlying NET formation differ considerably within these general pathways, depending on stimulant.<sup>46,47</sup> For instance, PMA-induced NET formation requires protein kinase C activation, calcium flux, ROS, myeloperoxidase (MPO) and NE but bacteria-derived ionophore-induced NET formation can work independently of protein kinase C activation, ROS, MPO, or NE.<sup>46</sup> Because NETs with a similar makeup can be induced by very different stimuli, pinpointing what triggers increased NET formation in a pathogenic manner is challenging in gut inflammation and IBD. For instance, disparate factors such as *C albicans* and PMA can both stimulate the release of NETs containing calprotectin, a known biomarker in IBD.<sup>4</sup> However, there may be other ways to pinpoint stimulants. Bacterial factors (eg, N-formylated peptides) may induce the release of different granule types and measuring the levels of primary vs secondary granule proteins such as the ratio of myeloperoxidase to lactoferrin in IBD, may provide insights into the types of neutrophil stimulants in general and therefore NET stimulants.<sup>48</sup>

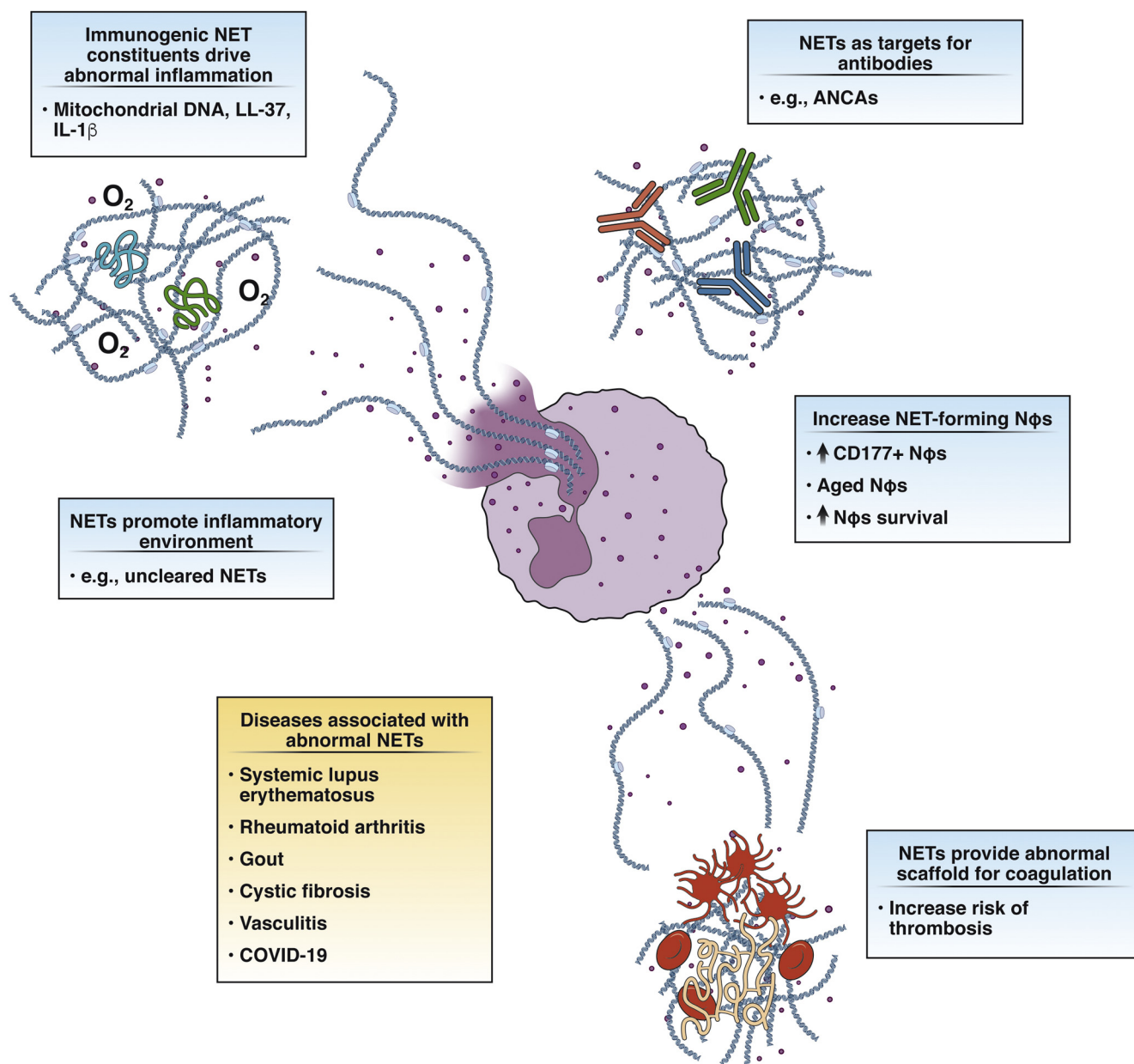
## NETS as a Pathological Factor in Human Disease

As with many host protective mechanisms, the deployment of NETs can be a double-edged sword.<sup>6,49</sup> NETs can promote or prolong both innate and adaptive immune responses in a wide variety of diseases (Figure 1). In cystic fibrosis, NETs directly induce IL-8 and TNF- $\alpha$  production by monocyte-derived macrophages,<sup>50</sup> and in atherosclerosis, NETs augment IL-1 $\beta$  and IL-6 production by monocytes stimulated with cholesterol crystals.<sup>51</sup> In rheumatoid arthritis (RA), NETs provide a scaffold for autoantibodies against citrullinated histones,<sup>52</sup> and in systemic lupus erythematosus (SLE), NETs containing LL-37 and high-mobility group box 1 can trigger plasmacytoid dendritic cell production of interferon  $\alpha$ .<sup>53</sup> NETs can also facilitate thrombosis in vasculitis by providing a framework for platelet and tissue factor adhesion.<sup>54</sup> NETs augment cancer progression by trapping and spreading cancerous cells and polarizing neutrophils toward an immunosuppressive N2-like population.<sup>55,56</sup> Finally, NETs contribute to sustained proinflammatory cytokine production, mucous secretion, and thrombosis in COVID-19 (coronavirus disease 2019).<sup>57,58</sup>

## NETS in IBD

### Increased NET Formation in IBD

To date, 8 studies (Table 1) have demonstrated an increased presence of NETs in the inflamed gut mucosa,

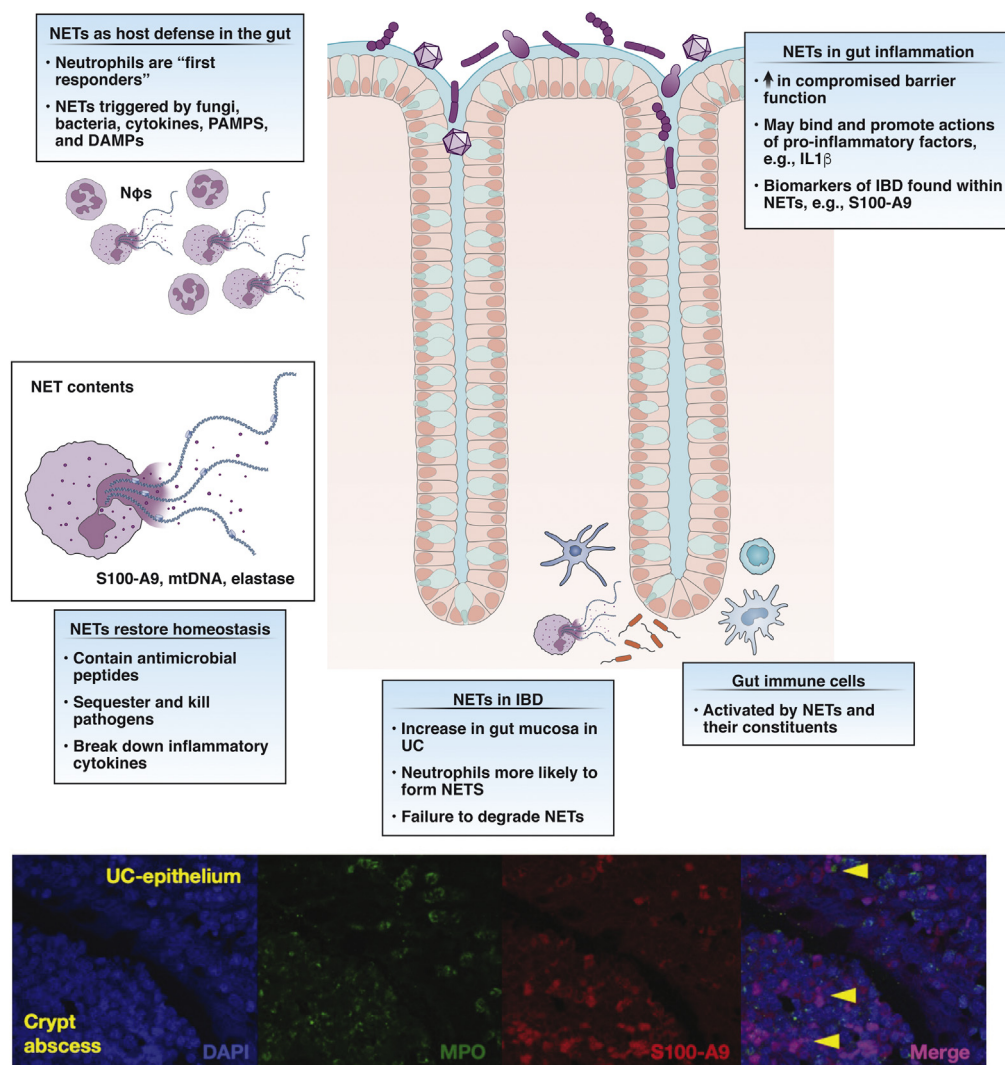


**Figure 1. Pathogenic processes of NETs in human diseases.** N $\phi$ s, neutrophils.

stool, or blood in IBD, 4 of which stipulate that NET abundance is positively correlated with active disease.<sup>64,66,69,70</sup> Liquid chromatography-mass spectrometry-based IBD proteomics studies reveal an increase in key NET proteins such as myeloperoxidase (MPO) and NE, both of which are highly specific to neutrophils and involved in chromatin decondensation during NET formation,<sup>71</sup> as well as increased calprotectin and cathepsin G, both of which have been found in NETs<sup>4</sup> in both intestinal biopsies<sup>72</sup> and fecal samples.<sup>73</sup> Increased levels of NET-associated proteins in IBD have also been demonstrated by immunofluorescence, immunohistochemistry, or Western blot in intestinal biopsies<sup>60,65,66,69</sup> as well as in the colonic mucosa of experimental colitis in mice.<sup>70</sup> Using Western blot, these increased NET-specific

proteins included NE, MPO, and citrullinated histone H3, another widely used NET biomarker,<sup>66,74</sup> and using immunofluorescence, NETs have been found as DNA complexed with NE, MPO, and histones.<sup>60,65,66,69</sup> Additionally, increased NET components have been found in the circulation of IBD patients<sup>69,70</sup> by capture enzyme-linked immunosorbent assay that measured DNA bound to MPO as a surrogate marker of a NET. Although these findings are important, colocalization or correlation of DNA with NET-implicated proteins is not outright evidence of NET formation because DNA of any source can bind proteins released from neutrophils through several processes including apoptosis and degranulation, and more rigorous qualification and quantification is suggested in Box 1.





**Figure 2. NETs in the gut and IBD.** Inflamed UC gut mucosa and the presence of NETs (colocalization of MPO and s100a9 in crypt abscesses and subepithelium [arrow-heads]). s100a9 is part of the calprotectin molecule. DAMPs, damage-associated molecular patterns; N $\Phi$ s, neutrophils; PAMPs, pathogen-associated molecular patterns.

Is increased NET formation a shared phenomenon in UC and CD? Several studies have reported this in both entities,<sup>60,64,69,70,73</sup> while others found no evidence in CD.<sup>65,66</sup> Different experimental methods and patient cohorts may explain some of the discrepancies. For instance, Dinallo et al<sup>66</sup> ruled out an increase in NETs in CD intestinal biopsies based on the absence of increased expression of peptidyl-larginine deiminase 4 (PAD4), a nuclear citrullinating enzyme classically believed to be essential for NET formation. However, it has been proven that PAD4 is not required for all forms of NET release, as shown with *C albicans* and group B *Streptococcus*-mediated NET formation.<sup>44,46</sup> However, as UC is typically associated with higher neutrophil recruitment,<sup>30,33</sup> if true, the finding of increased NETs in UC relative to CD is perhaps not unexpected.

Increased NET abundance is also not unique or specific to IBD and is likely present in any situation in which there is significant neutrophil involvement and gut microbial exposure. For instance, in necrotizing enterocolitis, in which gut barrier defense is markedly compromised by inflammation, high levels of NETs can be found in gut tissue.<sup>75</sup> However,

higher levels of proteins associated with NETs including MPO, NE, and calprotectin can differentiate individuals with IBD from those with noninflammatory conditions such as irritable bowel syndrome in fecal metaproteomic studies,<sup>73</sup> suggesting that increased NET production in the IBD is related to gut inflammation.

### Are Neutrophils in IBD More Likely to Form NETs?

Are neutrophils in IBD more prone to forming NETs? Several angles are noteworthy. First, the local biological microenvironment is crucial. For instance, in diabetes, a hyperglycemic environment primes NET formation by upregulating PAD4,<sup>76</sup> and in SLE, circulating microparticles derived from apoptotic cells can increase the potential for NET formation.<sup>77</sup> Serum from patients with UC and CD; and media from ex vivo culture of inflamed UC mucosa can stimulate higher spontaneous NET formation.<sup>64,65</sup> This suggests the involvement of inflammatory mediators in the circulation and

### Box 1. NETs in IBD: Experimental Considerations

To achieve better clarity in future work, several experimental considerations are noteworthy.

1. Multiple characterization of NET components beyond the use of single NET markers such as PAD4 are needed. Ideal methodology would combine the use of sandwich enzyme-linked immunosorbent assays for blood work, targeting DNA-MPO/citH3/NE complexes,<sup>59</sup> colocalization studies in tissue using immunohistochemistry,<sup>60</sup> intravital microscopy, and live cell imaging.<sup>61,62</sup>
2. For NET formation studies, neutrophils must be isolated with an appreciation of the ability of calcium, magnesium, and chelators to alter NET formation capacity.<sup>44,63</sup>
3. As for stimulants, PMA should ideally only be used as a positive control<sup>44</sup> and more physiologically relevant stimuli such as patient sera,<sup>64</sup> cultured medium from inflamed tissue cultures,<sup>65</sup> and combinations of IBD relevant cytokines<sup>66</sup> should be used.
4. Additionally, previous and current treatment regimens within patient cohorts should be stated in detail because several widely used IBD treatments such as infliximab<sup>66,67</sup> and mesalamine<sup>68</sup> have been shown to directly inhibit NET formation.

from the mucosa, but triggers by transmigrated gut microbial components such as bacterial DNA<sup>78–80</sup> are also likely stimuli.

Second, are there intrinsically distinct neutrophil phenotypes in IBD that are more prone to NET formation? Disease-specific neutrophil subsets such as type I interferon hyperresponsive neutrophils in SLE<sup>81</sup> or low-density neutrophils, implicated in a variety of diseases<sup>82,83</sup> that are considered more susceptible to form NETs, have been described. He et al<sup>64</sup> found that neutrophils, irrespective of whether they are obtained from UC, CD, or non-IBD subjects, had similar NET formation capacity when stimulated with IBD sera. Dinallo et al<sup>66</sup> demonstrated that in vitro NET formation capacity by active UC neutrophils is similar to healthy controls in response to a variety of stimulants including TNF- $\alpha$ , LPS, and PMA. However, 3 studies provided contrary evidence, stating that in vitro, neutrophils from CD and UC<sup>70</sup> or from UC alone<sup>65,69</sup> have a primed or intrinsically increased NET formation capacity, reported as spontaneous<sup>65,70</sup> or PMA induced.<sup>69</sup>

Of interest, NET-prone CD177+ neutrophils are found in increased proportions in the blood and inflamed intestinal mucosa of both UC and CD patients.<sup>22,84</sup> CD177 is a glycoprotein that is exclusively expressed on neutrophils, neutrophilic myelocytes, and metamyelocytes. CD177 is involved in neutrophil transmigration and is upregulated upon acute bacterial infection.<sup>85</sup> CD177+ neutrophils have a higher expression of granule protein genes<sup>86</sup> and are an activated or primed subset in several diseases including antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), SLE, and asthma.<sup>87,88</sup> In IBD, CD177+ neutrophils have a shifted gene expression profile toward microbial defense and release more ROS, MPO, and calprotectin, and produce more NETs than CD177- neutrophils.<sup>22</sup> Notably CD177+ neutrophils in inflamed mucosa of CD and UC patients positively correlate with Crohn's Disease Activity Index and UC Mayo Score.<sup>22</sup>

Third, are there increased numbers of aged neutrophils in IBD that are more likely to form NETs? Neutrophil lifespan can be prolonged by growth factors, cytokines, and microbial

products during infection and inflammation.<sup>89,90</sup> Delayed apoptosis leads to the persistence of autoantigens in RA and AAV,<sup>8</sup> enhanced risk of tissue damage in acute coronary syndromes,<sup>91</sup> and enhanced NET formation capacity in cystic fibrosis.<sup>50</sup> In IBD, spontaneous neutrophil apoptosis is delayed by IL-8 and granulocyte colony-stimulating factor.<sup>92,93</sup> With relevance to IBD, it has been reported that a systemic, aged, proinflammatory neutrophil population with higher NET formation capacity, defined as a CD62L<sup>lo</sup> CXCR4<sup>hi</sup> subset, can be driven by the gut microbiota via TLRs and myeloid differentiation factor 88-mediated pathways in mice.<sup>94</sup> This population is diminished upon depletion of the microbiota with antibiotics.<sup>94</sup>

### *Proinflammatory actions of NETs in IBD: Abundance vs Composition*

The composition of NETs and their specific effects is a further factor to consider above abundance alone. For instance, in psoriasis, NETs containing proinflammatory LL-37-DNA complexes activate plasmacytoid dendritic cells, leading to increased interferon alfa production.<sup>49</sup> In RA, autoantibodies against NET components such as citrullinated vimentin are found in the circulation leading to augmentation of IL-6 and IL-8 inflammatory responses.<sup>52</sup> and in SLE, NETs are embedded with highly proinflammatory oxidized mitochondrial DNA (mtDNA), which stimulates type I interferon signaling through the DNA sensor stimulator of interferon genes.<sup>95</sup>

In 2018, Angelidou et al<sup>65</sup> described bioactive IL-1 $\beta$ -decorated NETs in UC but not in CD. These IL-1 $\beta$ -containing NETs formed through an autophagy-dependent mechanism that facilitates the extrusion of NET contents.<sup>44</sup> Here, they showed that IL-1 $\beta$ -decorated NET production was linked to the expression of DNA damage inducible transcript 4 (DDIT4) in UC.<sup>65</sup> DDIT4 is an important, ubiquitously expressed stress-response mammalian target of rapamycin inhibitor linking cellular stress to autophagy-mediated NET-associated IL-1 $\beta$  responses in familial Mediterranean

**Table 1.** A Summary of NET Studies in IBD

| Reference                     | Methods  | Key findings  |
|-------------------------------|--|---|
| Bennike et al <sup>72</sup>   | Liquid chromatography-mass spectrometry (UC = 10 vs controls = 10).  | <ul style="list-style-type: none"> <li>NET-associated proteins increased in colonic mucosa: lactoferrin, MPO, NE, calprotectin, neutrophil defensin 3—UC.</li> </ul>  |
| Lehmann et al <sup>73</sup>   | Liquid chromatography-mass spectrometry (UC = 14, CD = 11, other = 29, controls = 17)  | <ul style="list-style-type: none"> <li>NET proteins increased in stool: NE, MPO, azurocidin, and cathepsin G—UC/CD.</li> </ul>  |
| Dinallo et al <sup>66</sup>   | Immunohistochemistry, immunofluorescence, Western blot; in vitro NET induction, DSS-induced colitis (UC = 9, CD = 9, controls = 12). | <ul style="list-style-type: none"> <li>Increased, colocalized PAD4, NE, MPO and citrullinated histone H3 expression in inflamed mucosa—UC.</li> <li>UC/control neutrophils similar NET formation capacity in vitro—UC.</li> <li>Infliximab treatment diminishes NETs—UC.</li> <li>PAD4 inhibitor attenuates colitis—DSS induced.</li> </ul>   |
| Li et al <sup>69</sup>        | Immunofluorescence, ELISA, in vitro NET induction; DSS-induced colitis (UC = 24, CD = 24, controls = 10).                            | <ul style="list-style-type: none"> <li>Increased MPO-DNA complexes in blood—UC/CD.</li> <li>NET formation capacity enhanced in vitro—UC.</li> <li>Impaired NET degradation in plasma—UC/CD.</li> <li>Increased, colocalized NE and citrullinated histone H3 in inflamed colonic mucosa—UC/CD.</li> <li>Increased NET deposition in colon, depleted by DNase I treatment—DSS induced.</li> <li>Procoagulant activity enhanced by NETs in vitro—UC/CD.</li> </ul> |
| Angelidou et al <sup>65</sup> | Immunofluorescence, Western blot; ELISA, in vitro NET induction (UC = 23, CD = 11, other = 15, controls = 25)                        | <ul style="list-style-type: none"> <li>Increased, colocalized NE and citrullinated histone H3 in biopsies—UC.</li> <li>Increased MPO-DNA complexes in blood—UC.</li> <li>Sera/ex vivo culture media enhances control NET formation in vitro—UC.</li> <li>IL-1<math>\beta</math> NETs mediated by autophagy via DDIT4/stress pathway in vitro—UC.</li> </ul>   |
| Gottlieb et al <sup>60</sup>  | Immunofluorescence (UC = 6, CD = 6, control = 2)   | <ul style="list-style-type: none"> <li>Increased, colocalized MPO, chromatin/histones and NE in biopsies—UC/CD.</li> </ul>  |
| Cao et al <sup>70</sup>       | Immunofluorescence, ELISA, in vitro NET induction, DSS colitis (UC/CD = 51).   | <ul style="list-style-type: none"> <li>Enhanced MPO-DNA complexes in blood—UC/CD.</li> <li>Enhanced NET formation in vitro—UC/CD.</li> <li>NETs enhance procoagulant activity in vitro—UC/CD.</li> <li>DNase I protects against DSS-induced colitis.</li> </ul>   |
| He et al <sup>64</sup>        | In vitro NET induction (UC = 28, CD = 23, controls = 12)   | <ul style="list-style-type: none"> <li>Sera enhanced patient/control NET formation in vitro—UC/CD.</li> <li>IgG from PR3-ANCA-positive IBD enhanced control NET formation in vitro—UC/CD.</li> <li>DNase I treatment decreased procoagulant activity in vitro—UC/CD.</li> </ul>   |

ANCA, antineutrophil cytoplasmic antibody; CD, Crohn's disease; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PR3, proteinase 3; UC, ulcerative colitis.

fever.<sup>96</sup> DDIT4 expression was higher in UC neutrophils and in control neutrophils stimulated with UC colon tissue culture media.<sup>65</sup> It was proposed that this DDIT4/NET/IL-1 $\beta$  pathway may be specific to UC (not CD) as a form of IL-1 $\beta$  autoinflammatory disease. Angelidou et al<sup>66</sup> previously described that in familial Mediterranean fever, neutrophils undergo autophagy-mediated NET formation via the DDIT4 pathway through stress mediators such as epinephrine. Studying the effects of similar stress mediators on NET formation may also be relevant in UC.

ANCAs may play a role in NET-mediated IBD pathology. ANCAs drive the development of AAV in which they target proteinase 3 (PR3) or MPO<sup>97</sup> and initiate neutrophil-rich necrotizing inflammation, causing organ damage.<sup>98</sup> In UC, the perinuclear type, pANCA, is found in up to 80% of UC patients,<sup>99,100</sup> with a distinct subset of UC-specific ANCAs, DNase I-sensitive ANCAs, known for targeting only nuclear-protein

complexes.<sup>99,101</sup> This UC-derived DNase I-sensitive ANCA can bind to antigens present on NETs.<sup>102</sup> In 2016, He et al<sup>64</sup> showed that NET formation is amplified when neutrophils were incubated with ANCA-IgG isolated from active PR3-ANCA-positive IBD patients. In addition, Li et al<sup>69</sup> proposed a further mechanism, in which ANCAs decreased the breakdown of NETs, by attenuating DNase I activity particularly during active IBD. Finally, ANCAs in IBD might activate neutrophils through Fc receptor stimulation when binding granule components such as PR3 or MPO, which have been translocated to their outer membrane during activation, as described with AAV.<sup>101,103</sup>

### Are There Proinflammatory Mitochondrial NETs in IBD?

Another area of significant interest in NET-driven pathology is the existence of NETs embedded with

**Table 2.** Summary of NET-targeted Therapies in IBD and Other Diseases

| Targeted NET component  | Inhibitors  | IBD Studies   | Non-IBD Studies  |
|---|---|---|--|
| Peptidylarginine deiminase, a nuclear citrullinating enzyme essential for some forms of NET formation | Chloramidine, BB-CI-amidine, neonatal inhibitory factor, streptonigrin  | Chloramidine—Reduced clinical signs and symptoms in DSS-induced colitis. <sup>116,117</sup><br>Streptonigrin—Reduced colonic inflammation, weight loss, and diarrhea; improved histological scoring in DSS-induced colitis. <sup>11</sup> | Murine models of lupus and MPO-ANCA-associated vasculitis. <sup>118–120</sup>                    |
| Neutrophil elastase—involved in chromatin decondensation during NET formation                         | Prolastin, elaspol, range of low-molecular-weight HNE inhibitors  | Elaspol—Reduced weight loss and histological score in DSS-induced colitis, reduced IL-17–based inflammation <sup>121,122</sup>  | Non-IBD—various including (cardio)pulmonary inflammatory diseases, RA, and cancer <sup>123</sup> |
| NET DNA   | Recombinant human DNase, DNase I  | DNase I—Reduced weight loss; lower disease activity index; lower histological score; reduced thrombotic tendencies; reduced IL-1 $\beta$ , IL-6, and TNF- $\alpha$ <sup>69,70</sup>   | Non-IBD—good safety profile in cystic fibrosis, moderately efficacious <sup>124</sup>            |
| IL-1 $\beta$ –decorated NETs  | Anakinra, rilonacept, canakinumab   | Anakinra (ongoing)—IASO phase II trial for ASUC. Question: Does antagonism of IL-1 signaling in addition to intravenous corticosteroid treatment improve outcomes in ASUC patients? <sup>125</sup>  | Various including RA, FMF, and hyperimmunoglobulinemia D syndrome <sup>126,127</sup>             |
| Other approaches  | <ul style="list-style-type: none"> <li>- Antibody-mediated targeting of NETs for macrophage degradation.<sup>128</sup> Not only breaks down DNA but also proinflammatory/destructive NET proteins.</li> <li>- Cross-linking of NET-inhibitory receptors such as signal inhibitory receptor on leukocytes-1.<sup>129</sup> Inhibits NETs but preserves intracellular bacterial killing.</li> </ul> |   |  |

ANCA, antineutrophil cytoplasmic antibody; ASUC, acute severe ulcerative colitis; DSS, dextran sulfate sodium; FMF, familial Mediterranean fever; IASO, Interleukin 1 blockade in Acute Severe Colitis; IBD, inflammatory bowel disease; IL, interleukin; MPO, myeloperoxidase; NET, neutrophil extracellular trap; RA, rheumatoid arthritis; TNF- $\alpha$ , tumor necrosis factor alpha.

mitochondrial DNA (mtNETs). This is relevant as an association of increased mtDNA levels with active UC and CD have been reported.<sup>104</sup> mtNET formation has been described as early, nonlytic, and ROS dependent and was originally demonstrated with neutrophils primed by granulocyte-macrophage colony-stimulating factor followed by TLR4 or complement receptor activation.<sup>105</sup> Here, NETs were found to contain mitochondrial DNA and the granule proteins NE and MPO but, interestingly, not genomic DNA. Yousefi et al<sup>55</sup> recently proposed mtNETs are an active, proinflammatory, antimicrobial tool that utilize mtDNA fragments no longer needed within the cell in combination with granule proteins, suggesting that they are true non-lytic NETs. Although the mechanisms remain unknown, mtNETs are now supported by many as an alternative form of NET release,<sup>5</sup> although they should be carefully differentiated from incomplete mitophagy, which can lead to the release of oxidized nucleoids containing mitochondrial components following cell damage.<sup>106</sup> Their similarities with bacteria point to mitochondrial components within mtNETs as potent inflammatory mediators<sup>107,108</sup> and oxidized mtDNA and mtNETs have now been implicated in a range of diseases, including SLE and chronic granulomatous disease<sup>95</sup> and in sterile injury, surgery,<sup>109</sup> and cancer.<sup>110</sup>

### NETs and Thrombotic Tendencies in IBD

One potential implication of increased circulating NETs in IBD is an increased thrombotic risk. Both UC and CD patients have an increased (3-fold) risk of thromboembolic events compared with the general population.<sup>111</sup> Although abnormalities in coagulation and fibrinolytic systems such as elevated circulating fibrinogen, prothrombin, and microparticles have been found, these abnormalities are insufficient to explain the increased thromboembolic risk in IBD.<sup>111</sup> Recently, 3 separate studies have linked NETs to increased thrombotic tendency in IBD.<sup>64,69,70</sup> In 2016, He et al<sup>64</sup> found that NETs contributed to increased coagulation, which could be counteracted by DNase I. Here, they highlighted that NET NE can inactivate tissue factor inhibitor and that NETs provide a scaffold for platelet and erythrocyte adhesion. The same group went on to demonstrate that incubating normal platelets with NETs from active IBD patients enhanced procoagulant activity by 32% and the ability to support fibrin formation by 42%.<sup>70</sup> Recently, they reported the treatment of a dextran sulfate sodium-induced mouse colitis model with DNase I led to lower disease activity and a decrease in prothrombotic effects.<sup>69</sup> Intriguingly, they also found ANCA-IgG to have a role, inducing NET formation as well as microparticle shedding by cultured endothelial cells, which accelerated



coagulation.<sup>8</sup> The effect of NETs on prothrombotic tendencies in IBD is a novel area and may have similarities to other diseases such as vasculitis.<sup>54,112</sup>

### Beneficial Roles of NETs in IBD

NETs have been shown to play a beneficial role in downregulating inflammation in several conditions. In RA, NETs can downregulate IL-6 and upregulate IL-10 secretion in LPS-activated macrophages.<sup>113</sup> In gout, NETs contribute to the resolution of inflammation directly by degrading cytokines and chemokines via serine proteases and when NET formation is impaired, the propensity of monosodium urate crystals, the trigger for gout, to activate neutrophils is amplified.<sup>114</sup> Proteases within NETs have been shown to be efficient regulators of cytokine activity in vitro and in vivo and will also play a role in regulating a large number of cytokines in IBD.<sup>115</sup> Finally, circulating NETs may have a role in clearing damage-associated molecular patterns such as transmigrated bacterial components,<sup>78–80</sup> a mechanism proven in murine sepsis.<sup>62</sup> Thus, some care must be taken in targeting NETs as a therapy in IBD.

### Conclusion

The knowledge of NET biology has allowed new insights into the inflammatory processes of many diseases. Although NET formation is a relatively unexplored area in IBD, it presents an excellent opportunity to understand the potential contribution of a major immune cell, the neutrophil, in the pathogenic and nonresolving gut inflammation in IBD. Importantly, the development of NET-targeted therapies in IBD can draw on lessons from other diseases (summarized in Table 2). Targeting NETs may form a novel treatment approach to achieving complete mucosal healing in IBD.

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#### Conflicts of interest

The authors disclose no conflicts.

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